

## Does ATP Cross the Cell Plasma Membrane?

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Although there is an abundance of evidence which indicates that ATP is released as well as taken up by cells, the concept that ATP cannot cross the cell membrane has tended to prevail. This article reviews the evidence for the release as well as uptake of ATP by cells. The evidence presented by various investigators clearly indicates that ATP can cross the cell membrane and suggests that the release and uptake of ATP are physiological processes.

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The importance of adenine nucleotides has long been recognized in the metabolic regulation of both catabolic and anabolic pathways and as the energy source for chemical and mechanical activity of cells [1]. Many of the metabolic systems that utilize adenine nucleotides are located in the cytosol, whereas it is the mitochondrion that synthesizes ATP during aerobic oxidative phosphorylation. The critical role of ATP in carbohydrate metabolism and tissue respiration [2], as an energy supplier for various intracellular reactions and in muscle contraction [3,4], is well recognized. Therefore ATP must cross intracellular membranes. However, the issue of whether or not ATP can get in and out of the cell was not adequately recognized until recently. The effects of ATP extracellularly and intracellularly have only recently come into prominence with the realization that ATP uptake by cells as well as release from cells can affect functions both outside and inside the cell. Let us review the evidence for the release and uptake of ATP.

### RELEASE OF ATP

There is a great deal of evidence indicating that nerve activity is accompanied by release of purines. Evidence for the release of ATP from sensory nerve endings was reported in 1953 [5]. Holton and Holton [5] were the first to demonstrate the presence of ATP in the effluent from a rabbit ear artery associated with sensory nerve stimulation. Prior to this, any physiological role of extracellular ATP had been regarded as highly unlikely, the general assumption being that an anion containing three negative charges could not possibly pass across the cell membrane [6-8]. However, with the development of sensitive and rapid methods of ATP detection, many studies have been performed since then and many species have been investigated.

Release of ATP from central nervous structures both *in vitro* and *in vivo* was subsequently reported [9-15]. In addition, there are reports of ATP release following nerve stimulation in various peripheral organs, including the subcutaneous adipose tissue, kidney, heart, nerve-diaphragm preparation, and lung [14-15]. The

evidence presented by these investigations clearly suggests that release of purines following nerve stimulation is a general phenomena. Although it has been shown that ATP release is secondary to neurotransmitter release [16], the question which could be raised is whether this release is due to ATP movement across membranes as a result of exocytosis. The studies of Israel et al. [17], however, have shown that ATP is released in a more specific manner by the post-synaptic membrane of the electric organ of the Torpedo in response to depolarization. Israel et al. [17] verified the post-synaptic site of ATP release by demonstrating that curare blocks the release of ATP. The above study [17] therefore demonstrates that ATP is released by a mechanism that does not involve release of synaptic vesicles and that the release is in response to a physiological stimulus following nerve stimulation.

In several of the studies cited above, ATP itself was detected. The question whether or not all the purines released are derived from intact ATP liberated from the cell will not be discussed here. Suffice it to state that a certain portion of the purines is detected extracellularly as ATP. The profound vasodilatory and circulatory regulatory effects of adenine nucleotides are also recognized to the extent that Green and Storer [18] once described ATP shock, i.e., ATP released from the ischemic cells produced shock.

Using a direct bioassay system for ATP, Boyd and Forrester [19] showed that ATP was released from contracting skeletal muscle *in vitro*. The possibility that ATP was released from muscle fibers damaged during dissection does not appear to be valid because that ATP was released under conditions in which  $K^+$  was not. Forrester [20] subsequently showed that ATP was released into the venous effluent from human forearm muscle during performance of a controlled amount of isometric exercise. These results were substantiated by Parkinson in 1973 [21] who noted an increase in blood levels of ATP, ADP, and AMP occurring as long as five minutes after whole body exercise. Paddle and Burnstock [22] showed an increase in the levels of ATP in the coronary sinus effluent of perfused guinea pig hearts when the myocardium was made hypoxic. It is not clear, however, from that study whether ATP came from the nerves, vascular smooth muscle, or myocardium. Forrester and Williams [23] subsequently showed that ATP was released from oxygenated cells and that ATP release was significantly increased within 30 seconds of rendering the cells hypoxic. They further showed that when hypoxic cells were transferred to an oxygenated state the level of ATP release reverted to the previous low levels. The amount of ATP released was calculated to fall well within the range necessary to produce maximum vasodilatation of the coronary vascular bed. Continuous release of ATP from oxygenated cells supports the possibility that the tone of coronary vessels could be a function of this release.

Clemens and Forrester [24] recently used isolated working rat heart preparation which was perfused with Krebs- $HCO_3$  buffer containing 11 mM glucose. Their results also showed that under aerobic conditions ATP was released into the coronary sinus effluent at a rate of 5.9 pmoles/min. When hypoxia was induced by using Krebs buffer bubbled with 95 percent  $N_2$ :5 percent  $CO_2$ , ATP release increased to 46.1 pmoles/min. These investigators concluded that a significantly greater amount of ATP is released from the myocardium during hypoxia and that the amount of ATP released under those conditions would produce a substantial vasodilatation if applied directly to the coronary vessels.

It has also been shown that cultured aortic endothelial or smooth muscle cells exposed to trypsin or thrombin selectively release a high proportion of their ATP [25]. Furthermore, it was shown that the ATP release was not due to a non-specific in-

crease in membrane permeability since the cells remained viable and there was very little release of cytoplasmic protein [25]. Although the precise mechanisms involved in ATP release remain unknown, it is possible that this release process could be in some respects similar to the selective release of prostaglandins from stimulated cells.

Pull and McIlwain [9] also showed that ATP was released from electrically stimulated brain slices. Wu and Phillis [26] have recently shown that the rate of basal release of ATP from rat sensory motor cortex was 1.43 femtomole/min/mm<sup>2</sup>; however, direct stimulation of the cortex with 5 mA pulses of 0.2 msec duration caused a 30-fold increase in ATP release. These as well as other investigators [15,26] have suggested that ATP could be a mediator in a "purinergic" system in the central nervous system. Thus, ATP is released both by exocytic and by molecular transmission through membranes.

### CONCLUSIONS CONCERNING ATP RELEASE

From the information presented above it is clear that active skeletal muscle, cardiac muscle, and brain tissue release significant concentrations of ATP into the extracellular space sufficient to profoundly affect local blood flow. A residual amount of ATP and other nucleotides may also reach the general circulation and indeed nucleotides have been detected in the human circulation as long as five minutes after whole body exercise. On the basis of this information Forrester [27] suggested that there exists a system of "metabolic communication" in the body mediated by circulating purine compounds. Regardless of whether or not this is true, the studies by these investigators clearly indicate that the release of ATP is a physiological process. The precise nature of ATP release, however, remains to be determined.

In addition to the experimental evidence presented above, which clearly indicates that ATP release and therefore the passage of ATP across the cell membrane occurs in various tissues, one might pose the following question from a teleological point of view. What is the reason for the presence of highly active ecto-ATPase on the surface of a large mass of skeletal muscle and especially vascular endothelium [28] in addition to the plasma ATPase and the ecto-ATPase on other cells? Clearly their known function is to degrade ATP. However, in order to perform that role, ATP has to be provided to those extracellular sites. Under normal as well as under adverse circulatory conditions ATP has to come from inside the cell to be available to the active ecto-ATPase on the surface of the large mass of skeletal muscle, in addition to the ecto-ATPase on other cells and the plasma ATPase but in doing so ATP has to cross the cell membrane as ATP.

### ATP AND CELL MEMBRANE PERMEABILITY

The studies of Trams [29] have shown that low concentrations of externally applied ATP produced a profound change in cellular permeability of monolayer cultures. His studies showed that K<sup>+</sup>-flux in cultured astrocytes increased briefly fivefold in the presence of  $6 \times 10^{-5}$ M ATP and that higher concentrations of ATP produced a prolonged permeability change. The studies of Rozengurt and Heppel [30] have shown that treatment of spontaneously or virus-transformed mouse cells with ATP causes a rapid and significant increase in membrane permeability. ATP treatment of such cells results in a massive efflux of nucleotide pools and of ions [30] and allows the entry of normally impermeable molecules such as p-nitrophenyl phosphate or NAD into the cells. These effects of ATP are reversible and the viability of the ATP-treated cultures is not impaired. This technique has been used to study the effect on glycolysis and the hexose monophosphate shunt of several

nucleotides and cofactors in whole cells [31]. The studies listed above therefore indicate that external ATP can regulate the cell surface permeability. Furthermore, it has been shown that the effects of exogenous ATP depend on the concentration of intracellular ATP, i.e., a decrease in intracellular ATP leads to a marked increase in the sensitivity of cells to external ATP [30]. Thus, it could be concluded that in some types of cells external and internal ATP have opposing effects. These findings may have considerable relevance to the regulation of passive permeability of membranes.

Recent studies of Diker et al. [32] have shown that the effects of external ATP on the cell surface (increased passive permeability) is completely independent of serum-derived proteins. They further showed that external ATP labelled the 44,000 dalton membrane protein and that this protein is directly involved in the regulation of membrane permeability. The potential significance of this possibility for the control of membrane permeability warrants further studies.

In contrast to transformed cells, inclusion of ATP into the external medium has been shown to decrease the leakage of a number of intracellular enzymes from human leukocytes and rat lymphocytes [33]. Furthermore, it has been shown that this protective effect of ATP is concentration-dependent, since at physiological concentrations the action is slight and relatively short-lived, whereas at higher concentrations it is more pronounced and persists for a longer period [33]. The studies described above [30,33] therefore indicate that external ATP produces opposite effects in transformed and untransformed cells. Whereas ATP produces a significant increase in membrane permeability of transformed cells, it causes membrane permeability to decrease significantly in the above untransformed cells. Although these studies indicate that membrane permeability is altered with external ATP, the exact molecular mechanism of the regulation of membrane permeability by ATP remains unknown.

### ATP UPTAKE BY TISSUES

Having dealt with the issue that ATP can get out of the cell, let us look at the evidence for the opposite phenomenon, i.e., ATP uptake by cells.

The belief that cell membranes are impermeable to ATP appears to have arisen as a corollary to a general theory that cell membranes are impermeable to anions [34]. Although a number of studies subsequently indicated that certain anions could in fact enter and leave the muscle cell [35–36], ATP was not considered to be one of these exceptions [6–8,34] and, in the absence of evidence to the contrary, this attitude has tended to prevail. Following the demonstration that externally added ATP induced contractions in isolated muscle fibers, Buchthal et al. [37] suggested that ATP had penetrated the cell membrane but this observation seemed to have been ignored. It is possible, however, that this was a surface effect of ATP. Nonetheless, in 1970 it was shown that  $^{14}\text{C}$ -ATP entered intact skeletal muscle cells *in vitro* and evidence was provided that the labelled ATP present intracellularly was due to the transport of ATP itself from the incubation medium and not due to the formation of ATP from its breakdown products [38]. Despite that and other evidence presented in 1976 [39], the notion that tissue cells are impermeable to ATP has tended to prevail [6,7]. It is therefore appropriate to review the evidence for the uptake of ATP by muscle cells.

In the study described above [38] intact soleus muscle from rats was incubated in Krebs- $\text{HCO}_3$  buffer containing 10 mM glucose, 5 mM 8- $^{14}\text{C}$  ATP and 5 mM  $\text{MgCl}_2$ . Following one hour incubation the muscles were homogenized in TCA-HCl mixture and the medium as well as the tissue extracts were then subjected to electrophoresis.

The individual nucleotide spots were detected under ultraviolet light and counted for the radioactivity in each fraction. The concentrations of adenine and hypoxanthine nucleotides in medium and tissue were calculated from the radioactivity observed in each nucleotide fraction. A nucleotide was considered to have an intracellular distribution when the total tissue contents exceeded the extracellular content. Extracellular concentrations were calculated on the assumption that the concentration in the extracellular water was the same as that of the medium. In parallel experiments, extracellular space was determined using raffinose as an extracellular marker. The distribution of  $^{14}\text{C}$ -labelled ATP between medium and tissue clearly provided the indication that external ATP entered the muscle cell [38]. It is important, however, to discuss whether the labelled ATP within the cell had an intracellular distribution and whether the labelled ATP within the cell entered as ATP and was not synthesized from inosine or adenosine. If one assumes that the concentration of isotopically labelled nucleotides in the extracellular water is the same as that of the external medium, then on the basis of the concentration in the medium shown in the previous paper [38] it is possible to calculate the amount of radioactive nucleotide present in the extracellular space. If the amount of radioactive nucleotide found in the tissue exceeds the extracellular value, one may assume that this excess is present intracellularly, that there is an accumulation of the nucleotide in the extracellular space, or that the ATP is attached to the cell membrane. However, because of the extensive degradation of external ATP it is difficult to believe that the observed accumulation of ATP occurred in the extracellular space. Moreover, depending on whether the  $^{14}\text{C}$ -labelled ATP or ADP was used, the ratio of labelled ATP to labelled ADP within the muscle varied from 6.5:1 to 0.9:1. When an ATP regenerating system was used to maintain the ATP level in the incubation medium, more labelled ATP was found within the cell and the ATP:ADP ratio increased to 89:1. If ATP did not enter the muscle cell as ATP, one would not have expected to see the dramatic change in the intracellular ATP:ADP ratio depending on whether or not one maintains higher ATP levels in the medium during incubation. Moreover, the dramatic increase in the intracellular ATP:ADP ratio in the presence of an ATP regenerative system would tend to rule out the possibility that labelled intracellular ATP arose by synthesis from adenosine. Using  $^{14}\text{C}$ -labelled adenosine it was shown that the contribution of this synthetic pathway under those experimental conditions was negligible [38]. When labelled ADP was used in the medium both  $^{14}\text{C}$ -ATP and ADP were found within the cell, in approximately equal amounts. This, however, contrasts with the preponderance of labelled ATP observed when  $^{14}\text{C}$ -ATP served as a substrate and indicates that both ATP and ADP can enter the cell. It could also be argued, however, that AMP rather than ATP entered the cell and was converted to ATP and that this was the cause for the observed labelled ATP within the cell. However, labelled AMP was found to be restricted to the extracellular compartment [39], thereby eliminating the possibility that the presence of labelled ATP within the muscle was due to the entrance of labelled AMP.

It could also be argued that the extracellular space values observed in those studies [37] may have been an underestimation of the true extracellular space. If that was the case externally added ATP could have been confined to the extracellular compartment rather than having an intracellular distribution. This possibility is extremely unlikely since the extracellular space values observed (0.25 ml/g) are similar to the *in vitro* value reported previously [39]. Moreover, the extracellular space would have had to be 75 percent of the total tissue space if ATP were confined to an extracellular compartment. An extracellular space of 75 percent is extremely hard to

accept, particularly for an intact muscle preparation, such as the soleus muscle. Thus, it could be concluded that the ATP present in those studies [38] was indeed intracellular and that ATP itself crossed the cell membrane.

It is well known that certain hormones and phlorizin which bind to the cell membranes without entering the cells have larger distribution volumes than extracellular water. Thus, it could be suggested that ATP binds to a specific membrane binding site and concentrates on the surface of the cell rather than entering the cell itself. It is unlikely, however, that the same is the case with ATP due to the fact that (a) extensive degradation of external ATP occurs in the presence of tissues, (b) the volume of distribution of ADP was much less than that of ATP, and (c) external AMP failed to have an intracellular distribution [38,40]. If ADP as well as AMP were restricted to the extracellular space, then it could have been suggested that there is a specific binding site on the cell surface for ATP. However, the fact remains that external ADP also had an intracellular distribution but its distribution was significantly less than that of ATP. This, coupled with the fact that external AMP did not have an intracellular distribution, clearly suggests that ATP binding to the cell surface could not be the explanation for the proposed entry of this nucleotide into the cell.

It could be argued, however, that ATP binds to a specific membrane binding site and that the binding affinity for ATP is significantly greater than that for ADP. If this were the case, the ATP could be binding to the cell membrane in the above experiments [38] rather than having an intracellular distribution. Although this possibility cannot be completely ruled out with the above experiments, the fact remains that active degradation of ATP occurs in the presence of the muscle, which would suggest that ATP binding to the cell surface is an unlikely explanation for the entry of ATP into the cell. In additional studies a "double isotope" method was employed to follow the nucleotide activity [41]. Muscles were incubated with ATP labelled with 8- $^{14}\text{C}$  and with  $^{32}\text{P}$  in the alpha phosphorus position. The results indicated that the ratio of  $^{32}\text{P}$  to  $^{14}\text{C}$  in the muscle was the same as that in the incubation medium. If the ratio of  $^{14}\text{C}$ : $^{32}\text{P}$  in the cell was not the same as that in the incubation medium, it would have raised doubts concerning intracellular uptake of  $\text{A}\bar{1}\text{P}$ . However, since the ratio of  $^{32}\text{P}$ : $^{14}\text{C}$  was the same in the medium and muscle, it provides further evidence that both  $^{14}\text{C}$ - and  $^{32}\text{P}$ -labelled ATP had entered the tissue to the same extent [41]. In that study [41], however, along with ATP and ADP the presence of inosine and adenosine in incubation medium as well as in the tissues was observed when tissues were incubated in the presence of  $^{32}\text{P}$  ATP and 8- $^{14}\text{C}$  ATP. Since the  $^{32}\text{P}$  ATP was labelled in the alpha (ribosyl) position, the presence of  $^{32}\text{P}$  in the inosine or adenosine spots of the electrophorotogram would not be expected unless there was some contamination of  $^{32}\text{P}$  ATP. In view of this we have repeated the ATP uptake by the rat soleus muscle using  $\alpha$ - $^{32}\text{P}$  ATP and the results confirmed the presence of intracellular  $^{32}\text{P}$  ATP and ADP in amounts similar to the ones we reported previously [41]. However, in contrast to previous results,  $^{32}\text{P}$  was not detected with inosine or adenosine in the medium or the muscle. We are therefore left to conclude that the presence of  $^{32}\text{P}$  inosine and adenosine in the muscle and the medium previously [41] must have been due to some contamination. The fact that intracellular ATP levels were similar to the ones observed previously [41] would indicate, however, that whatever contaminant was present in the previous study it did not affect our observations concerning ATP uptake by rat tissues. Thus, it could be concluded that if one incubates intact tissues in a medium containing ATP, ATP has an intracellular distribution. Moreover, the results indicate that by maintaining higher medium ATP levels the relationship between medium ATP and ATP uptake

was not linear. The  $^{14}\text{C}$ -ATP uptake activity was at a near saturation level when medium ATP levels were maintained above  $1\ \mu\text{mole/ml}$ . Since the occurrence of saturation in uptake activity with increasing medium substrate concentration indicates the involvement of a membrane carrier or a limiting number of cell surface binding sites in the uptake process, the results presented previously [40,41] suggest that ATP uptake by tissues could be a carrier-mediated process. Although the above studies clearly indicate that ATP enters the intact tissue cells, the precise localization and distribution of ATP within the cell remains to be determined.

The studies by Ziegelhoffer et al. [42] have demonstrated that small amounts of exogenous ATP (1 mM) but not ADP or AMP increased the ATP and the total adenine nucleotides of the hypoxic myocardium. Williams et al. [43] have also shown that addition of ATP but not ADP or adenosine to the medium caused an increase of ATP contents of the cultured myocardial cells. These studies therefore indicate that ATP itself but not its breakdown products increase intracellular ATP levels. Recent studies of Maxild [44] have provided evidence for renal uptake of externally added ATP. He found that with ATP in the medium, the degradation of carbohydrates by rat kidney cortical slices was decreased (i.e., conversion of glucose to  $\text{CO}_2$ ). The addition of AMP to the medium, on the other hand, stimulated renal degradation of glucose. Thus, if AMP or adenosine was responsible for decreasing glucose degradation by kidney slices, then the results should have been similar irrespective of whether ATP or AMP was used. The fact that these two compounds demonstrated opposite effects clearly suggests that the effects of ATP are not mediated by AMP or adenosine. Moreover, Maxild [44] found that addition of probenecid, an inhibitor of the classical organic anion transport system in the kidney, induced a pronounced reduction of the renal uptake of  $^{14}\text{C}$  ATP. On the basis of his experiments, Maxild [44] concluded that ATP is taken up into the renal cells by the transport system of organic anions. Using squid giant axon, Pant et al. [45] found that the same set of proteins were phosphorylated in the axoplasm regardless of whether the ATP was applied intracellularly or extracellularly. These results therefore indicate that ATP in the extracellular space is utilized by some ATP translocation mechanism in the process of intracellular phosphorylation. Measurements of the apparent influx of ATP across the squid axon membrane yielded results consistent with the view that ATP in the extracellular fluid could be transported into the axoplasm. The studies of Ayad and Hughes [46] have indicated that the adenylate cyclase is located on the intracellular surface of the plasma membrane of mouse lymphoma cells. They showed that when ATP was added to the incubation medium, cyclic AMP levels of the mouse lymphoma cell line increased. This effect of ATP was not mediated by adenosine, nor was it enhanced by NaF. On the basis of their results, Ayad and Hughes [46] also concluded that ATP enters the cells and acts as a substrate for adenylate cyclase. Thus, a great deal of evidence has been presented by various investigators which clearly indicates that ATP can enter the cell as ATP.

Since the general consensus still may be that adenosine rather than ATP crosses the cell membrane, we attempted to see whether we could dissociate ATP uptake from prior metabolism to adenosine. For such studies, rat hepatocytes were incubated in Krebs- $\text{HCO}_3$  buffer containing glucose, glutamate, pyruvate, and 8- $^{14}\text{C}$ -labelled ATP or adenosine. The results showed that uptake from 3 mM ATP was markedly inhibited by the 5'-nucleotidase inhibitor,  $\alpha,\beta$ -methylene ADP (AOPCP), and by dipyridimole, indicating that there is an adenosine-dependent component of ATP uptake in isolated hepatocytes. Addition of 3 mM probenecid, an inhibitor of organic anion transport, inhibited  $^{14}\text{C}$  uptake from ATP but did not

inhibit uptake from adenosine. Furthermore, after the adenosine-dependent component of ATP uptake had been inhibited by the addition of AOPCP and dipyridimole, probenecid exhibited further inhibitory effects on the remaining portion of the uptake. These results therefore indicate clearly that there is an ATP uptake component in the hepatocytes which is independent of prior metabolism to adenosine [47].

Despite the abundance of evidence presented by various investigators for ATP uptake by cells, it would be useful to demonstrate this by more direct means so that the issue of ATP uptake can be resolved once and for all. Autoradiography is a possible means of demonstrating the intracellular localization of exogenously applied ATP. However, with the present techniques it is not possible to distinguish between ATP and its breakdown products by autoradiographic means. It may be possible to use labelled ATP analogues which are not subjected to degradation and autoradiography to pinpoint the precise localization of ATP uptake site(s) in the cell. However, prior to conducting such experiments, it has to be determined that the ATP analogues are transported into the cell by the same mechanism as ATP itself. There is some preliminary evidence which indicates that ATP translocation is associated with the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (unpublished observations). However, much more work needs to be carried out before the system which transports ATP into and out of the cell is characterized. In view of the evidence presented above, it could be concluded that the release as well as the uptake of ATP is a general phenomenon and that ATP can indeed cross the cell membrane.

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